Cytotoxic and Antibacterial Activities of Constituents from *Calophyllum ferrugineum* Ridley

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Abstract: This study evaluated the chemical composition of *Calophyllum ferrugineum*, cytotoxicity against human breast cancer (MCF-7) and human lung carcinoma (A-549) cell lines as well as antibacterial activities against two Gram-positive bacteria, *S. aureus* and *B. subtilis* and two Gram-negative bacteria, *P. aeruginosa* and *E. coli*. Phytochemical investigations of the bark extract yielded isoapetalic acid (1), apetalic acid (2), 6-hydroxy-2-methoxyxanthone (3) and ent-epicatechin (4). Meanwhile, betulinic acid (5), protocatechuic acid (6) and amentoflavone (7) were isolated from the leave extract. Isoapetalic acid (1) and apetalic acid (2) exhibited cytotoxic activities towards both cancer cell lines and both Gram-positive bacteria. Compounds (3-7) were inactive or showed moderate activities towards cytotoxic and antibacterial tests. This study presents the first report on the phytochemicals investigation from *C. ferrugineum* and all compounds are reported for the first time from this source.

Keywords: *Calophyllum ferrugineum*; cytotoxic activities; antibacterial activities. © 2016 ACG Publications. All rights reserved.

1. Plant Source

*Calophyllum* is one of the genera from Guttiferae family and normally used as timber. *Calophyllum ferrugineum* is an evergreen tree that grows in lowland or colline mixed dipterocarp forest up to 30 meters tall. It can be found around the Southeast Asia especially in Malaysia. The bark is used traditionally as a decoction for a mother after childbirth [1]. This paper describes the first isolation and characterization of pure chemical constituents from the barks and leaves of *C. ferrugineum*. The cytotoxic and antibacterial activities of all chemical constituents were also evaluated. The plant was collected from Lenggong, Perak with a voucher specimen number
SK2587/14. The plant was identified by Dr. Shamsul Khamis and deposited at the herbarium of Universiti Putra Malaysia.

2. Previous Studies

A bioactivity-guided isolation of the ethyl acetate extract of an endophytic fungus 9PR2 isolated from internal root tissue of C. ferrugineum was reported [2]. The extract of 9PR2 was tested for cytotoxic effect against murine leukaemic cell line P388 by MTT assay. It led to the isolation of seven known polyesters identified as (+)-6-hydroxymellein, 15G256V, 15G256α-1, 15G256α-2, 15G256n, 15G256α, and 15G256β from the fungi based on dereplication process using HPLC.

3. Present Study

The air-dried barks (2.70 kg) and leaves (2.30 kg) of C. ferrugineum were macerated with dichloromethane, ethyl acetate, and methanol for three days respectively. The extracts were filtered and concentrated under reduced pressure to yield dichloromethane bark extract (127.16 g, 9.79%), ethyl acetate bark extract (8.86 g, 0.33%), methanol bark extract (208.40 g, 7.72%), dichloromethane leave extract (65.91 g, 2.87%), ethyl acetate leave extract (21.40 g, 0.93%) and methanol leave extract (175 were mixed and washed with cold hexane: EtOAc, 9:1) to afford 406 subfractions. Fractions 85 – 105 were pooled together to give isoapetalic acid (1) (187 mg, 9.35%) as yellow gum [3] while fractions 230 – 440 were combined to yield apetalic acid (2) (242 mg, 1.37%) as yellow gum [4].

The ethyl acetate bark extract (8.86 g) was fractionated by VLC eluted with n-hexane-CHCl3-EtOAc in increasing polarity by 10% to give seven major fractions. The purification of the fifth fraction (0.22 g) by CC (n-hexane: EtOAc, 9:1) gave 200 subfractions. Further purification of subfractions 78 – 150 (23.5 mg) by sephadex LH-20 eluted with MeOH in isocratic mode yielded 6-hydroxy-2-methoxylxanthone (3) (2.9 mg, 0.03%) as a pale yellow amorphous [5]. Compound (3) was previously reported as synthetic compound and this is the first time it was isolated from natural sources. The sixth fraction (0.36 g) was subjected to CC (n-hexane: EtOAc, 9:1) to give 500 fractions. The purification of subfractions 460 – 495 by sephadex LH-20 with MeOH in isocratic mode and solidification in the mixture of n-hexane and EtOAc furnished ent-epicatechin (4) (165 mg, 1.87%) as a pale brown amorphous [6, 7].

The fractionation of the dichloromethane leave extract (50 g) with VLC using n-hexane-CHCl3-EtOAc as eluent systems in increasing polarity by 10% gave seven major fractions. The sixth fraction (0.85 g) was purified by CC (n-hexane: EtOAc, 9:1) to afford 406 subfractions. Subfractions 131 – 175 were mixed and washed with cold n-hexane to produce betulinic acid (5) (130 mg, 0.25%) as white solid [8]. The ethyl acetate leave extract (20 g) was fractionated by VLC with n-hexane/EtOAc as eluents in 10% stepwise gradient to yield nine major fractions. The seventh fraction (0.86 g) was subjected to CC (n-hexane:EtOAc, 3:2) to furnish protocatechuic acid (6) (36 mg, 0.18%) as a yellow needle [9]. The purification of the eighth fraction (2.27 g) over CC (n-hexane:EtOAc, 3:7) gave amentoflavone (7) (381 mg, 1.91%) as yellow amorphous [10].

Cytotoxic activity: The cytotoxic activity was evaluated by MTT colorimetric assay. The percentage of cell viability for both cell lines after treatment with all compounds for 24 hours was compared with the untreated control cell. The IC₅₀ value acted as a parameter for a cytotoxic activity where it indicated 50% cell inhibition by the compound. Isoapetalic acid (1) and apetalic acid (2) gave similar IC₅₀ values against A-549 cell lines at 249.04 and 249.43 μM, respectively (Table 1). In contrast, apetalic acid (2) was more toxic towards MCF-7 cell lines with IC₅₀ values 100.36 μM compared to isoapetalic acid (1) at 151.60 μM. However, the mechanism exerted by both compounds towards both cell lines was not investigated in this study. It has been reported that other derivatives of chromanone carboxylic acid such as calophenic acid, brasiliensic acid, and inophylloidic acid, previously isolated from C. inophyllum were found to exhibit significant cytotoxic activities against KB cell line with IC₅₀ values between 9.7 – 11.0 μM [11]. It signifies the potential of chromanone carboxylic acid
derivatives unique to *Calophyllum* species to be further investigated as a potent cytotoxic agent. Meanwhile, amentoflavone displayed cytotoxic activity against MCF-7 cell lines at IC$_{50}$ 176.80 µM. This finding was supported by a study that reported amentoflavone showed significant cytotoxicity against MCF-7 at 150 µM and as a potent apoptosis-inducing agent in MCF-7 via mitochondria-dependent pathway [12]. On the other hand, *ent*-epicatechin (4) and protocatechuic acid (6) displayed more than 80% cell viability at the highest concentration tested thus, were devoid to the cytotoxic test of both cell lines.

![Chemical structures](image)

**Table 1.** Cytotoxic activity of the tested samples.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MCF-7</th>
<th>A-549</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC (%)$^a$</td>
<td>IC$_{50}$ (µM)$^b$</td>
</tr>
<tr>
<td>1</td>
<td>26.64 ± 0.74$^c$</td>
<td>151.60 ± 4.30</td>
</tr>
<tr>
<td>2</td>
<td>3.79 ± 1.02$^c$</td>
<td>100.36 ± 1.07</td>
</tr>
<tr>
<td>3</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>98.98 ± 1.51$^c$</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>56.76 ± 1.94$^c$</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>94.95 ± 0.64$^c$</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>47.29 ± 0.59$^c$</td>
<td>176.80 ± 2.43</td>
</tr>
</tbody>
</table>

$^a$ Cell viability at 100 µg/mL (highest concentration tested) as mean ± SD of triplicate experiments; $^b$ IC$_{50}$ is defined as the concentration where the cell viability is reduced to 50% compared to the control group. IC$_{50}$ is reported in µM unit; $^c$ The result is statistically significant different compared to control, $p < 0.05$; NT = Not tested; ND = Not Determined

**Antibacterial activity:** The antibacterial activity of all compounds was determined by minimum inhibition concentration (MIC) in serial broth microdilution. Two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis* and two Gram-negative bacteria, *Pseudomonas aeruginosa* and *Escherichia coli* were employed for this study. The tetrazolium (INT) colorimetric microplate assay method was chosen as it enhances sensitivity and accuracy of MIC determination since the formation of violet formazan derivatives by living bacteria can be quantified and yield greater reproducible result [13]. The MIC result as tabulated in Table 2 displayed isoapetalic acid (1) and apetalic acid (2) that exhibited antibacterial activities against Gram-positive bacteria, *S. aureus* and *B. subtilis* both at 31.25 µg/mL. According to Rios [14], pure compounds with MIC value < 100 µg/mL is considered to be active. No inhibition was observed against *P. aeruginosa* and *E. coli*. It indicates that both compounds were more selective towards *S. aureus* and *B. subtilis*, Gram-positive
bacteria tested in this study. The presence of n-pentyl with carboxylic acid moiety at end-terminal substituent in the structure increases the lipophilic character of the compound. Gram-positive microorganisms have low lipid content in the cell wall, thus, permit the high activity of more lipophilic compounds to reach their active site causing its disruption [15]. The previous report on the isolation of six chromanone acids from C. brasiliense demonstrated moderate-to-strong antibacterial activity especially to Gram-positive bacteria [16], thus, supports the findings. The structure activity study of a series of lipophilic N-acyldiamines towards antibacterial activity showed that the best results obtained were from compounds having 10 – 12 carbons alkyl chains [17]. Both flavonoids; ent-epicatechin (4) and amentoflavone (7) showed moderate inhibition towards all bacteria in the range of 62.5 – 500 µg/mL.

Table 2. Minimum inhibition concentration (MIC) of the tested samples.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Gram positive</th>
<th>Gram negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. aureus</td>
<td>B. subtilis</td>
</tr>
<tr>
<td>1</td>
<td>250</td>
<td>31.25</td>
</tr>
<tr>
<td>2</td>
<td>31.25</td>
<td>25.12</td>
</tr>
<tr>
<td>3</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>5</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>6</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td>Streptomycin Sulphate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.5</td>
<td>1.56</td>
</tr>
</tbody>
</table>

<sup>a</sup> Minimum inhibition concentration (µg/mL) in triplicate; <sup>b</sup> Streptomycin sulphate as standard control; NT = Not tested

Acknowledgements

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Supporting Information

Supporting Information accompanies this paper on [http://www.acgpubs.org/RNP](http://www.acgpubs.org/RNP)

References


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